JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1991, p. 753–756
0095-1137/91/040753-04$02.00/0

Vol. 29, No. 4

Francisella tularensis-Induced In Vitro Gamma Interferon, Tumor Necrosis Factor Alpha, and Interleukin 2 Responses Appear within 2 Weeks of Tularemia Vaccination in Human Beings

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Received 11 May 1990/Accepted 16 January 1991

Cell-mediated immunity is essential for protection against the intracellular bacterium *Francisella tularensis*, which causes tularemia. Positive in vitro T-cell responses in the form of lymphocyte proliferation and lymphokine interleukin 2 (IL-2) and gamma interferon (IFN-γ) secretion are found in memory immunity. Studies on the secretion of lymphokines with regard to the developing immunity to *F. tularensis* have not been published. Therefore, 14 subjects with no clinical history of tularemia were vaccinated with a live *F. tularensis* vaccine strain. The in vitro responses of five subjects (antigen-induced mononuclear cell and whole blood culture DNA synthesis and cytokine secretion) were measured twice a week throughout the period from 0 to 35 days after vaccination, and the peripheral blood lymphocyte subpopulations of nine subjects were determined between days 0 and 14. Positive reactions, i.e., responses exceeding those on day 0, were reached on day 10 with regard to the whole blood culture DNA synthesis response and IL-2 and IFN-γ secretion and on day 14 with regard to the mononuclear cell DNA synthesis response and tumor necrosis factor alpha (TNF-α) secretion. No measurable IL-4 was found in either the immune or nonimmune supernatants. Since the secretion of TNF-α was related to immunization, this points to the specificity of the phenomenon, even though the type of secreting cell is not yet known. If it is shown later that specific T cells produce it, the TNF-α response and the negative IL-4 finding may speak for the importance of the Th1-like pattern in immunity to *F. tularensis*.

Francisella tularensis, a small gram-negative bacterium, causes sporadic or epidemic infections known as tularemia. *F. tularensis* biovar palaearctica causes infections in Finland and northern Europe in general which are milder than those caused by the biovar tularensis. Tularemia is an intracellular infection, i.e., the bacterium survives in mononuclear phagocytes when the cells are in a nonactivated state. Cell-mediated immunity therefore confers protection against the microbe, as has been shown clearly in animal experiments (for a review, see reference 22). The in vitro T-cell responses observed in connection with human natural or vaccine-induced immunity include, in addition to positive T-cell proliferation, specific secretion of the lymphokines interleukin 2 (IL-2) and gamma interferon (IFN-γ), expression of IL-2 receptor-positive cells, and the possibility of generating *F. tularensis*-specific T-cell clones (8, 9, 21).

The above-mentioned studies focused on established (i.e., memory) immunity to *F. tularensis*. However, it is not known when during the development of immunity the in vitro responses emerge or whether the various responses appear simultaneously. These questions were addressed in this study by monitoring the development of IFN-γ and IL-2 responses, compared with the better characterized proliferative response, after vaccination of *F. tularensis*-naive subjects. In addition, we measured *F. tularensis*-induced tumor necrosis factor alpha (TNF-α) and IL-4 secretion, for which there are no previous data. For this purpose, 14 subjects were vaccinated with a live *F. tularensis* vaccine. Five of these were monitored during the 35 days after vaccination for cytokine secretion and lymphocyte proliferative responses, and in another nine subjects blood lymphocyte subpopulations were studied during the 14 days after vaccination.

MATERIALS AND METHODS

Subjects. Intradermal vaccination with *F. tularensis* live vaccine strain BB IND 157.6111, obtained from the U.S. Army Medical Research Institute for Infectious Diseases (Fort Detrick, Md.), was performed twice. First, nine voluntary subjects (age, 37 ± 12 years; six female and three male healthy laboratory workers) were vaccinated, and their peripheral blood lymphocyte subsets were determined from samples taken on days 0, 3, 7, 10, and 14 after vaccination. Two years later, a second arm of our study included five voluntary subjects (age, 35 ± 3 years; four female and one male healthy laboratory workers) who were vaccinated, and their cytokine secretion and DNA synthesis responses were monitored by means of samples taken on days 0, 3, 7, 10, 21, and 35 postvaccination. Informed consent was obtained from all the subjects.

***F. tularensis* antigen.** The antigen was Formalin-killed whole-cell antigen prepared from the vaccine strain as described earlier (21), and was used for both DNA synthesis and cytokine secretion measurements at a final protein concentration of 1.0 μg/ml, which has been found to be optimal.

Measurement of DNA synthesis. DNA synthesis was determined by using both mononuclear cell (MC) and whole-blood (WB) cultures (8). RPMI 1640 supplemented with 5% fetal calf serum was used as the culture medium. MCs were separated with a Ficoll-Isopaque gradient. A total of 50,000 MCs were cultured in a total volume of 200 μl. The WB

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TABLE 1. *F. tularensis*-induced and spontaneous DNA synthesis responses

Type of DNA synthesis	Median (range) cpm[a] (n = 5) on day postvaccination:						
	0	3	7	10	14	21	35
MC							
+ FT ag[b]	797 (628–2,000)	1,316 (655–2,255)	1,014 (796–1,374)	1,487* (1,216–2,970)	5,236** (2,552–12,600)	7,574** (2,105–16,544)	8,453** (2,321–12,192)
− FT ag	1,037 (466–1,773)	1,346 (362–2,486)	1,168 (847–1,622)	1,542* (1,081–2,592)	2,754** (2,081–2,884)	1,856 (676–2,655)	1,009 (778–1,723)
WB							
+ FT ag	109 (70–462)	91 (64–274)	129 (70–427)	2,676* (172–4,169)	1,603** (172–4,675)	8,019** (986–43,817)	6,250** (1,220–33,206)
− FT ag	126 (82–179)	131 (122–400)	132 (72–271)	77 (40–183)	135 (66–409)	55 (28–514)	204 (62–234)

[a] Antigen-induced and spontaneous values for each day were compared with the respective values on day 0 by using the one-tailed Mann-Whitney U test (*, $P \leq 0.05$; **, $P \leq 0.01$).
[b] FT ag, *F. tularensis* antigen.

cultures were performed in volumes of 200 μl after the final blood dilution of 1:16. Both cultures included three wells with *F. tularensis* antigen and no stimulant (spontaneous cultures). The culture period was 6 (MC) or 7 (WB) days, with [^{3}H]thymidine being present for the last 24 h. The median value of triplicate identical wells was taken as the result.

Preparation of supernatants and measurement of cytokines. MCs in a final concentration of 1.5×10^6 cells per ml were cultured with *F. tularensis* antigen (1.0 μg/ml) or with medium alone (to test spontaneous secretion) in a total volume of 1.6 ml for 2 days (IL-2 and IL-4 testing) or in a volume of 0.8 ml for 5 days (IFN-γ and TNF-α testing). The supernatants were centrifuged and stored at −20°C until tested.

IL-2 was quantified by using an IL-2-dependent mouse cytotoxic T-cell line (8). A total of 5,000 cytotoxic T-cell line cells in a 100-μl volume were cultured for 48 h with 100-μl aliquots of supernatant dilutions of 1:1 and 1:2. Triplicate cultures were used for each supernatant, and the median result of a 1:1 dilution was taken. The results are expressed as the counts of test cells stimulated by the supernatant IL-2. Several dilutions of a standard IL-2 preparation (Lymphocult-T-HP; Biotest, Frankfurt, Federal Republic of Germany) were also included to show the response of test cells. The number of counts per minute of test cells with *F. tularensis* antigen in the medium was 32.

IFN-γ was measured with an enzyme immunoassay prepared by us (1). Supernatants were tested individually. The detection limit of the test was 5 U/ml.

TNF-α and IL-4 were tested by using commercial enzyme immunoassay kits (TNF ELISA [Endogen, Boston, Mass.] and Intertest-4 [Genzyme, Boston, Mass.]). Supernatants were tested individually and according to the instructions given by the manufacturer. The sensitivities of TNF-α and IL-4 tests were 60 and 45 pg/ml, respectively.

Lymphocyte subsets. Mononuclear cells of samples taken on days 0, 3, 7, 10, and 14 were stained by using the conjugated monoclonal antibodies anti-Leu-4 (CD3), anti-Leu-3a (CD4), anti-Leu-2a (CD8), anti-IL-2 receptor (CD25) (Becton Dickinson, Mountain View, Calif.), and anti-B cell (B1) (Dakopatts, Glostrup, Denmark). The cells were incubated with each antibody for 30 min at 4°C, washed with phosphate-buffered saline (PBS) and resuspended in 10% glycerol in PBS. The percentage of fluorescent cells of a total of 200 cells was determined by using a Leitz UV microscope.

Statistical methods. The median values at the various intervals after the vaccination were compared with the initial value, i.e., that for day 0, and the differences were tested by using the Mann-Whitney U test.

RESULTS

MC and WB culture DNA synthesis responses. Antigen-induced MC DNA synthesis became positive on day 14, i.e., the median response differed statistically from the day 0 response (5,263 versus 797 cpm, $P < 0.01$) and remained positive for the study period ($P \leq 0.01$) (Table 1). Positive WB culture synthesis was already visible on day 10 (2,676 versus 109 cpm, $P < 0.05$) and thereafter ($P < 0.01$). A spontaneous synthesis response differing from the day 0 response could be found in the MC cultures from days 10 and 14 (day 10, $P = 0.05$; day 14, $P < 0.01$). The spontaneous response on day 10 explains the counts found in the antigen culture (1,487 cpm). There was no spontaneous synthesis in the WB cultures at any time (P value not significant for each day).

Cytokine secretion in vitro. (i) Antigen-induced IL-2 secretion appeared on day 10 (449 versus 29 cpm, $P = 0.01$) and continued in the rest of the samples (422, 239, and 812 cpm; $P \leq 0.01$) (Fig. 1).

(ii) An antigen-induced IFN-γ response appeared on day 10 (23 versus <5 U/ml, $P < 0.05$) and was positive in the remaining samples (35, 90, and 37 U/ml; $P < 0.01$) (Fig. 1).

(iii) The amount of TNF-α found in the cultures with *F. tularensis* antigen was higher on days 14 and 21 than on day 0 (400 and 500 versus 90 pg/ml, $P < 0.05$). An increase in secretion was also found on day 35, but it did not reach statistical significance (300 versus 90 pg/ml, $P < 0.10$) (Fig. 1).

(iv) The amounts of IL-4 in all supernatants were below the detection limit of the test, i.e., less than 45 pg/ml (data not shown).

Statistical significances, as shown above, between the antigen-induced responses on each day and those on day 0 were estimated. When comparisons were made between the *F. tularensis*-induced and spontaneous responses on each sampling day, essentially the same values were obtained. Some of the differences in MC DNA synthesis and IL-2 data were less significant, while the WB DNA synthesis and IFN-γ responses were parallel and the TNF-α responses showed more significant differences.

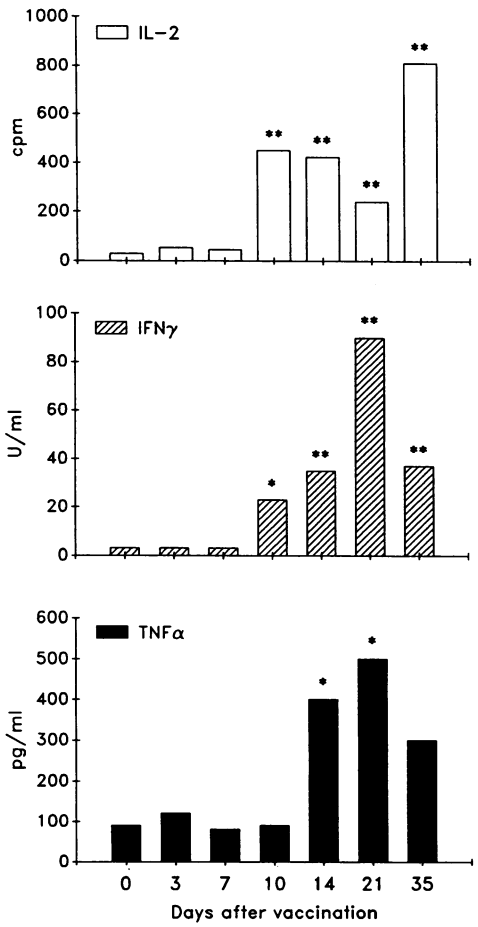

FIG. 1. Median *F. tularensis*-induced cytokine secretion responses ($n = 5$). The values for each day were compared with the respective values on day 0 (**, $P \leq 0.01$; *, $P \leq 0.05$). The spontaneous values were comparable to those on day 0 (data not shown).

Stimulation indices of each subject. When the five T-cell parameters (MC and WB DNAs, IL-2, IFN-γ, and TNF-α) were calculated in the form of stimulation indices (antigen-induced response divided by spontaneous response), all the indices for each subject became positive (stimulation index, >2) between 7 and 35 days and generally remained positive. The indices were highest in the samples from day 21 and day 35. The ranges for the day 35 indices, for instance, were the following: 2.3 to 11 (MC DNA synthesis), 5.6 to 535 (WB DNA synthesis), 2.6 to 38 (IL-2), 2.6 to 30 (IFN-γ), and 2.2 to 3.7 (TNF-α) (data not shown elsewhere).

Blood lymphocyte subpopulations. The percentages of CD3+, CD4+, and CD8+ cells and the ratios of CD4+ to CD8+ cells remained the same after vaccination (data not shown). The number of CD25+ cells, i.e., IL-2R+ cells, increased on days 7, 10, and 14 (median, 5, 8, and 6% versus 2% on day 0; $P < 0.01$ to 0.001). The median number of B cells was higher on days 3, 7, and 14 (5, 5, and 5% versus 3% on day 0; $P < 0.05$ to 0.001).

DISCUSSION

The results show that the vaccination-induced immune response to *F. tularensis* involves the appearance of in vitro lymphocyte proliferation (as measured by DNA synthesis) and secretion of the cytokines IL-2, IFN-γ, and TNF-α. The responses of all five subjects became positive with respect to all these variables, even though the stimulation indices were not persistently high in all of them. Most of the individual responses were nevertheless clearly distinguishable from the initial ones. The approach used here differs from our earlier studies (8, 9) with regard to the state of immunity. In earlier studies, the immunity was established as to its nature, i.e., memory immunity, while here we measured developing parameters. Our finding on the parallel appearance of IL-2 and IFN-γ responses may indicate that secretion of these cytokines takes place in the same T cells. Specific T-cell clones, induced by *F. tularensis* antigen, have been found to secrete both cytokines (21), but the information obtained by clones does not necessarily cover the responses of all the responding blood lymphocytes. Different kinetics in positive responses between IL-2/IFN-γ and TNF-α were found.

In earlier studies, positive T-cell proliferative and cytokine secretion responses were found only in immunized subjects (8, 9), while the control subjects had negative responses. In this study, the subjects were their own negative controls before the appearance of responsiveness. Therefore, no other controls were included. The constant association of positive blood MC responsiveness with the known immunization and vice versa, together with the finding that T-cell clones react positively to *F. tularensis* and negatively to the control bacterial antigens used (20), gives strong support for the specificity of these responses.

It was found previously that the proliferation measurement with WB cultures gives slightly different results from those of MCs (8). Here we found spontaneous proliferation in the MC cultures on days 10 and 14 but not in the WB cultures. Proliferation may have been due to the IL-2 receptor-positive lymphocytes present at that time. In contrast, the WB cultures also had autologous serum. According to our experiences, there is hardly ever spontaneous proliferation in the WB cultures, which may be related to inhibitory factors or cytokines present in human sera.

Positive TNF-α secretion reflected immunization. It is not known whether monocytes or T cells were responsible for its production, but if the monocytes were responsible, a signal would be needed from the immune T cells. IFN-γ did not seem to be a sufficient signal, since it became positive earlier than the TNF-α response. An increasing amount of evidence that T cells secrete TNF-α in situations of mitogenic or allogenic stimulation (2, 4, 12, 13, 17, 19) has emerged, and it is possible that *F. tularensis*-specific T cells produced TNF-α in our cultures. Complex interactions between TNF-α and T-cell proliferation/activation on the one hand (6, 11, 14–16, 23) and between TNF-α and monocyte/macrophage activation on the other hand (3, 7) have been reported.

Recently, CD4-positive T cells have been classified into Th1 and Th2 subsets, which are described as having separate functions and patterns of cytokine secretion. Th1 cells are considered to be responsible for delayed-type hypersensitivity reactions and macrophage activation and have been shown to secrete IL-2, IFN-γ, IL-3, TNF-α, and TNF-β, while the cytokine pattern of Th2 cells includes IL-3, IL-4, IL-5, IL-6, and TNF-α (10, 18). The cytokine findings, e.g., the positive secretion of IL-2, IFN-γ, and TNF-α (previous findings and this study), and the negative IL-4 results of this study emphasize the role of the Th1-like arm in *F. tularensis*-specific cell-mediated immunity.

The immunization process was reflected in an increasing number of IL-2 receptor-positive cells, possibly indicating

the expansion of *F. tularensis*-specific T cells. The proportions of peripheral blood CD3+, CD4+, and CD8+ cells remained stable, and the significance of the differences found in the numbers of B cells is unknown.

Measurements of cytokines, either from culture supernatants or directly from body fluids, offer new tools which should advance our understanding of protective immunity as well as pathological mechanisms of inflammation. For example, both positive and negative effects of TNF-α have been reported in studies of bacterial and viral infections (for a review, see reference 5). Our new finding that *F. tularensis* induces TNF-α from leukocytes in vitro as a result of vaccination with *F. tularensis* implies that this cytokine could be involved in protection against *F. tularensis*. On the other hand, *F. tularensis* can cause long-standing fever and pneumonia and its biovariant tularensis can even cause fatal infections in humans, and thus the production of TNF-α may also (or instead) contribute to morbidity in tularemia. Further studies on the production kinetics and levels of TNF-α and other cytokines in the sera of acutely infected patients may be useful to clarify this. On the other hand, it will probably be necessary to use an experimental animal model to establish the actual significances of the cytokine responses found in in vitro studies.

ACKNOWLEDGMENTS

We thank Rauni Kemi and Marja Suorsa for their excellent technical assistance. We also thank Toini Berzins of the University of Stockholm for helping us to establish the cytotoxic T-cell line cultures.

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